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TITLE: ANALYSIS OF SAXITOXIN FROM URINE USING CONTINUOUS-FLOW
FAST ATOM BOMBARDMENT & ELECTROSPRAY IONIZATION MASS
SPECTROMETRY

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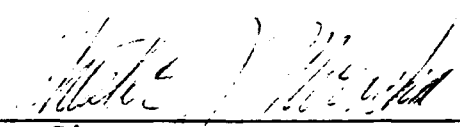
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KINETIC STUDY OF THE EXCRETION OF SAXITOXIN IN RATS
TREATED WITH SUBLETHAL AMOUNTS OF SAXITOXIN

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SUMMARY

A method for analysis of saxitoxin (STX) in urine of rats treated with STX was developed using CBA columns for clean-up of the extract and hydrogen peroxide oxidation prior to analysis by HPLC. Detection of STX is linear between 0.1 and 5 ng when measured by either peak height or area. It was used for analysis of urine from rats treated with 1 ug total STX. The limit of detection of pure standard is 100 pg.

Saxitoxin was found in the urine of the treated rat (1 ug total dose) as soon as 10 min after treatment and up to 8 hrs after dosing. In general, the excretion pattern was bimodal, i.e. the largest amount was found 1 hr after treatment, followed by a decline 2 and 3 hrs afterward, and then a reappearance of larger amounts at 6 and 8 hrs.

Adult female rats tolerate a 1 ug dose of STX given by intraperitoneal injection but succumb to 3 to 4 ug (i.p.) within 3 min.

INTRODUCTION

Saxitoxin (STX) was first isolated and described from toxic Alaska butter clams known by the Latin binomial *Saxidomus giganteus* (Chantz et al., 1957). The paralytic shellfish poisons (PSP) include STX which is one of the most serious marine toxins known (Hashimoto and Noguchi, 1989). Ingestion can cause paralysis often terminating in death. *Protogonyaulax catenella* produces STX and grows to a high density under favorable environmental conditions. The organism is ingested by bivalves which, when consumed, may in turn poison humans (Sommer, 1937).

Various analytical methods have been devised including high performance liquid chromatography (HPLC) separation (Sullivan and Wekell, 1987), capillary zone electrophoresis (CZE) [Thibault et al., 1991a and b], continuous flow fast atom bombardment (CF/FAB), [Caprioli, 1989], and ion spray mass spectrometry (IS/MS) and [Quilliam et al., 1989]. Perhaps the most useful and inexpensive is the HPLC method described by Lawrence et al., (1991) where STX was oxidized with hydrogen peroxide prior to separation on the HPLC column and detection by fluorescence. The limit of detection is 100 pg and the limit of sensitivity of the method is about 100 ppb. The oxidation procedure takes 10 min and analysis by HPLC another 10 min. This method adapts itself to analysis of biological fluids and is the primary determinant of success in this study.

There are numerous reports on the effects of STX and ion transport channels but little to none on metabolism in living animals or detection in the urine of treated animals. As an

example, Xia and Haddad (1993) studied the neuroanatomical distribution and binding properties of STX in the rat brain but offered no metabolic data. They concluded that adult rat brain has multiple sodium channels but similar binding affinities for all of them i.e. little selectivity. Fan and Makielski (1993) studied STX blockage of sodium channels in cardiac myocytes in the rat but no metabolism studies were reported. The study of binding sites and STX "dwell time" in the animal is important for studies of metabolism because STX can be found in the urine almost instantly (as fast as one can collect the urine) after insult. The questions remain as to what absolute amount is needed to bind and affect the rat and whether there is an activated metabolic species that actually binds to the receptor.

Our objectives in this study were (i) to study the detection of STX in the rat, using urine as the primary determinant, in order to verify exposure to STX; (ii) to identify any metabolic products that may be present other than the parent metabolite for purposes of monitoring exposure; and (iii) to develop a method of extraction and detection of STX in urine.

MATERIALS AND METHODS

Collection of rat urine after dosing with STX: The rats used in the experiment are Sprague Dawley, female, 9 to 10 months old and weighing 300-350 grams. Three rats were dosed with saxitoxin dihydrochloride (STX.2HCl) using water as the carrier solvent. One microgram of STX in 1 ml water was injected into the intraperitoneal cavity at zero time and then once more after 24 hours. The

rats survived the treatment and urine was collected after 1, 2, 3, 4, 6 and 8 hours on the first and second day treatments. On the third day, the rats were dosed once more but this time with 4 micrograms. All rats died within 3 minutes after administration of the third dose. Urine was collected within ten minutes from the bladder and stored in a freezer. Urine from nontreated rats (controls) was collected in the same manner and kept frozen.

Oxidation procedure for the analysis of STX by HPLC: Analysis of STX using the old method of postcolumn oxidation and detection by HPLC as described by Sullivan and Wekell (1987) is cumbersome. An alternative method is precolumn oxidation as described by Lawrence et al. (1991) in which the oxidation is performed under controlled conditions in sample preparation prior to injection into the HPLC apparatus. Two oxidation procedures were described, periodate and hydrogen peroxide. The method of choice for STX oxidation is hydrogen peroxide.

The method described by Lawrence et al. (1991) involves oxidation of STX at room temperature with hydrogen peroxide. The rat urine sample was first prepared by passing through a CBA column followed by concentration and oxidation.

CBA column procedure: The CBA column was purchased from Analytichem International, Los Angeles, CA. It is a carboxymethyl (hydrogen form) sorbent and a weak cation exchanger. Its pKa is 4.8 and thus it is useful for resolution of STX. The CBA bed volume is 100 mg; it is conditioned with 4 ml of methanol followed by 4 ml of

0.05 M $\text{KH}_2\text{PO}_4/\text{Na}_2\text{PO}_4$ (pH 8.0). To 0.1 ml sample of urine, 2 ml of 0.05 M $\text{KH}_2\text{PO}_4/\text{Na}_2\text{PO}_4$ (pH 8.0) was added, thoroughly mixed and loaded unto the CBA column. The column was washed with 5 ml water followed by 2 ml of methanol. The STX fraction was eluted off the column with 2 ml of 20 % aqueous acetic acid. The eluate was taken to dryness under nitrogen without heating.

Oxidation with hydrogen peroxide: The dried sample was dissolved in 100 μl of water to which was added 25 μl of aqueous hydrogen peroxide (10% w/v) and 250 μl of 1 N NaOH. The sample was mixed thoroughly and allowed to react for 5 min at room temperature (20 C). A 25 μl volume of concentrated acetic acid was added and mixed thoroughly. Twenty microliters of the solution was injected into the HPLC (Shimadzu) system.

Detection by HPLC: The instrument used was a Shimadzu SLC-6A (Shimadzu Co. Kyoto, Japan); C18 reverse phase (Waters NOVA-PAK) 75 mm x 3.9 mm i.d. detector with a Shimadzu RF-530 fluorescence monitor (excitation wavelength of 330 nm and monitoring wavelength of 410 nm). The HPLC mobile phase consisted of A = 0.1 M ammonium formate in water (adjusted to pH 6 with acetic acid) and mobile phase B = 20 % acetonitrile in mobile phase A (pH 6). Solvents A and B were run from 0.1 to 14 min with A at 1-0.7 ml/min and B at 0-0.3 ml/min. The retention time of STX on the column was 9.35 to 9.45 min. The chromatographic procedure was run at room temperature i.e. 23 to 25 C.

Percent recovery: experiments were done by spiking 0.1 ml rat

urine with 0.1 ml aqueous solution containing 100 ng STX. The latter was mixed with 2 ml buffer at pH 8.0 and loaded onto the column. The recovery was 95.5%. In another experiment, 2 ng of STX was spiked into 0.1 ml rat urine and then mixed with 2 ml of buffer (pH 8) and loaded onto the column. The recoveries in 2 experiments were: 100% and 110%; average 105%.

Standard curve: One-tenth milliliter of standard STX solutions (20 ng/ml, 200 ng/ml and 1 ug/ml) were mixed with 25 ul of aqueous H₂O₂ (10%), 250 ul 1N NaOH (react for 5 min at room temperature) and 25 ul acetic acid (concentrated) to stop the reaction. Twenty microliters of this mixture was injected into the HPLC. The latter injection volume gave 0.1, 1.0 and 5.0 ng oxidized STX shown in figure 1.

RESULTS AND DISCUSSION

Oxidation of STX: The fluorescent single oxidation product of STX as well as oxidation products of other Paralytic Shellfish Poisons (PSP) were described by Janecek et al. (1993). Quilliam et al. (1993) described the mass spectrometry of the oxidation product (protonated molecular ion in FAB is 296) and its fragmentation product (m/z 235). A standard curve based on fluorescence of the oxidized product is shown in figure 1. The oxidation product is stable to least one-half hour; we inject the mixture into the HPLC after 5 min. There is a linear relationship between the three

concentrations tested i.e. 0.1, 1.0 and 5 ng. The limit of detection is ~100 pg. This is truly amazing because it approaches the 15 pg limit of IS/MS reported by Thibault et al. (1991a and 1991b) and 50 pg by Mirocha et al. (1992). An overlay chromatogram showing the detector response (fluorescence) to various amounts (0.1, 0.5, 1.0 and 5.0 ng) is shown in figure 2. The same linearity is shown in the detector response as in the peak height plot. The detector response of the 5 ng injection has saturated the detector and affected the retention time (delayed). Note the unknown component present at Rf 4.5 in the standard. The unknown may be a STX derivative isolated with the parent compound or a by-product of the peroxide oxidation. It is always present in a constant ratio (10:1) to STX both in the standard and the rat urine isolation products and is shown figure 3.

Detection of STX in urine of adult rats dosed with STX: The collection of urine from live rats is a difficult art and often unpredictable as to the amount that can be collected at any one time. The alternative is to insert an indwelling catheter, which in itself is traumatic to the animal and may compromise metabolism and function of the excretory system. In these experiments, the amount of urine collected from each rat varied sometimes due to the excretion volume and other times due to accidents in collection. The urine was collected in a clean Petri plate by gently holding the rat in the hand over the plate and softly squeezing the mid section of the rat. The collection technique was unique and was done exclusively by one member of our group who had exceptional skills and patience at performing the operation. The collection

time is noted in each table and figure. The urine was frozen immediately and stored for 1 to 2 weeks before analysis. The pH of the urine varied between 6.0 and 6.5. STX is stable at this pH and will tolerate freezing.

Rats 5, 6 and 7 (weight ~300-350 g) received a total dose of 1 ug STX per rat (i.p.) on days 1 and 2 and tolerated this dose very well i.e., there were no external signs of trauma. However, 3 or 4 ug STX resulted in death within 3 min. The LD50 (i.p.) in an adult rat is 8 ug/kg whereas by stomach intubation it is 200 ug/kg for a young rat and 500 ug/kg for an adult rat. In estimating the quantitative data with STX, one is always left with the question of purity because the sample size available is small and difficult to quantitate.

Urine of rats 5, 6 and 7 was collected on day 1 at 1,2,3,4,6 and 8 hrs after dosing. The rats were dosed once more at 24 hrs and the same collection schedule was followed as on day 1. The quantitative results (expressed in nanograms per milliliter) are shown in table 1. In general, the majority of STX was found in the 1 hr post treatment urine (average of 182 ng/ml) followed by a decrease at 2 and 3 hrs (average of 22 ng/ml) and then an increase at 4 and 6 hrs (average of 64 ng/ml) i.e. bimodal excretion pattern. Rats 6 and 7 were dosed with 4 ug STX on day 3 and they succumbed within 3 min. It is important to note that STX was found in their urine (average of 631 ng/ml) almost instantly after injection. Such large concentration of STX in the urine soon after injection suggests that attempts at elimination or clearance is made instantly.

The HPLC tracing of the oxidized product of control rat urine is shown in figure 4-A. The peak at retention time 10.0 is an unknown component present in control rat urine. Figure 4-B shows the HPLC tracing of the oxidized STX product (retention time 9.7) in rat urine as well as the unknown component at 10.0. The two components are shown in an overlay of the two chromatograms (figure 4-C) to show the difference in retention time of STX and the unknown. The two are easily resolved and do not present a problem in detection and quantitation.

The total STX, expressed in nanograms, is shown in table 2. The data suggest that from a total of 1 ug injected on day 1, 236 ng or ~25% was recovered in the urine. On day 2, 91 ng or 9% was recovered. Rats 6 and 7 were injected with 4 ug on day 3; 0.07% of the dose was recovered at time of death i.e. 3 minutes after exposure.

A graphic representation of the data shown in table 1 is displayed in figure 5, where the average concentration of STX is normalized in units of nanograms/milliliter (ppb) for all 3 rats. The day 1 presentation of data shows the bimodal curve; however, data from the second injection on day 2 do not show an immediate excretory response as on day 1 but do show an increment rise in concentration from 2 to 4 hrs. The same pattern of excretion is shown in rat 7 (figure 6) indicating that perhaps once the animal is insulted with STX, the detoxification mechanism is compromised and does not react at the same speed with the second dose. The

best agreement in recovery of STX is found in rats 5 (figure 7) and 7 (figure 6) where 284 and 226 ppb respectively were found. The amount of STX found in rat 6 (figure 8) was 13 ppb, which is less than the others; however, although smaller, the pattern of excretion or ratio is similar. All rats show a decline at the 2 and 3 hr collection and then an increase at 4 hrs. The above data can also be expressed in absolute amounts detected (before normalization) in each rat; the latter is presented in table 2.

The concentration of STX (ng/ml) found in the urine of rats 6 and 7 dosed with 4 ug STX on day 3 of the experiment is shown in figure 9. The animals died 3 to 5 min after injection, although STX could be found in the urine almost immediately, i.e. the time that it took to make the collection. The speed at which STX is excreted is significant and suggests that a rapid detoxification mechanism exists in the rat.

There are at least two important and significant findings in this report: (i) it is possible to find STX in urine of the rat up to 8 hrs after dosing with as little as 1 ug of STX per adult rat and (ii) the kinetics of STX recovery suggests an initial excretion of the toxin into the urine, followed by release at hours 4 to 8. The latter is a bimodal excretion pattern suggesting an interaction within the rat. Presently we have no evidence for metabolism of STX by the female rat. This may be in part due to the lack of resolution of peroxidation products, formed or not resolved, or because peroxidation prevents discrimination between derivatives such as neosaxitoxin. An example of this is given by the report of

Lawrence et al. (1991) where the N-1-hydroxylated toxins (neosaxitoxin, B-2, GTX-1 and C-3) do not form fluorescent compounds with hydrogen peroxide oxidation whereas they do with periodate oxidation. On the other hand, STX, B-1, GTX-2, GTX-3, C-1 and C-2 form highly fluorescent derivatives with both periodate and hydrogen peroxide. Additional experiments should be done with periodate oxidation; however, the sensitivity of the N-OH derivatives with periodate is 5 to 10 times less sensitive than the hydrogen peroxide derivatives.

The recovery of the STX in urine 8 hrs after dosing is encouraging in that the potential for detection, depending on the dose used, could be extended up to 12 and 24 hrs. This is important in verification of STX in humans where STX poisoning is suggested. It may have application for detection of STX in victims of STX poisoning due to ingestion of contaminated mussels or in clandestine poisoning as in biological warfare. Additional experiments with the detection of STX in blood plasma or serum are warranted in that PSP may circulate in the blood system for significant periods similar to ochratoxin in human blood.

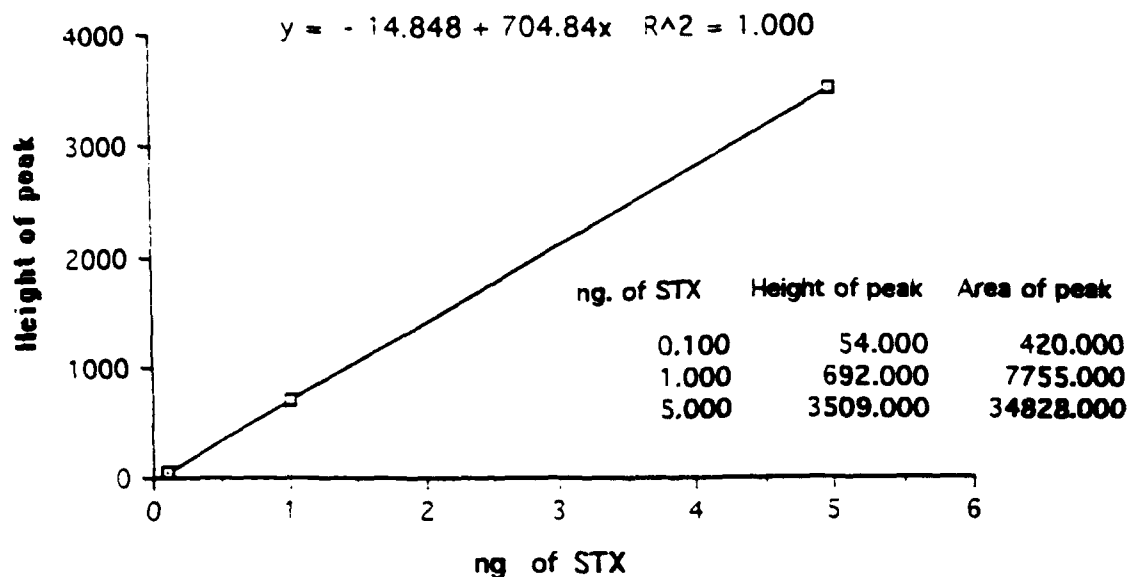


Figure 1. Standard curve, based on peak height, of saxitoxin after peroxide oxidation, resolution on a C18 column by HPLC and detection by fluorescence. The limit of detection is 100 pg. The actual peak height and area values are in the inset.

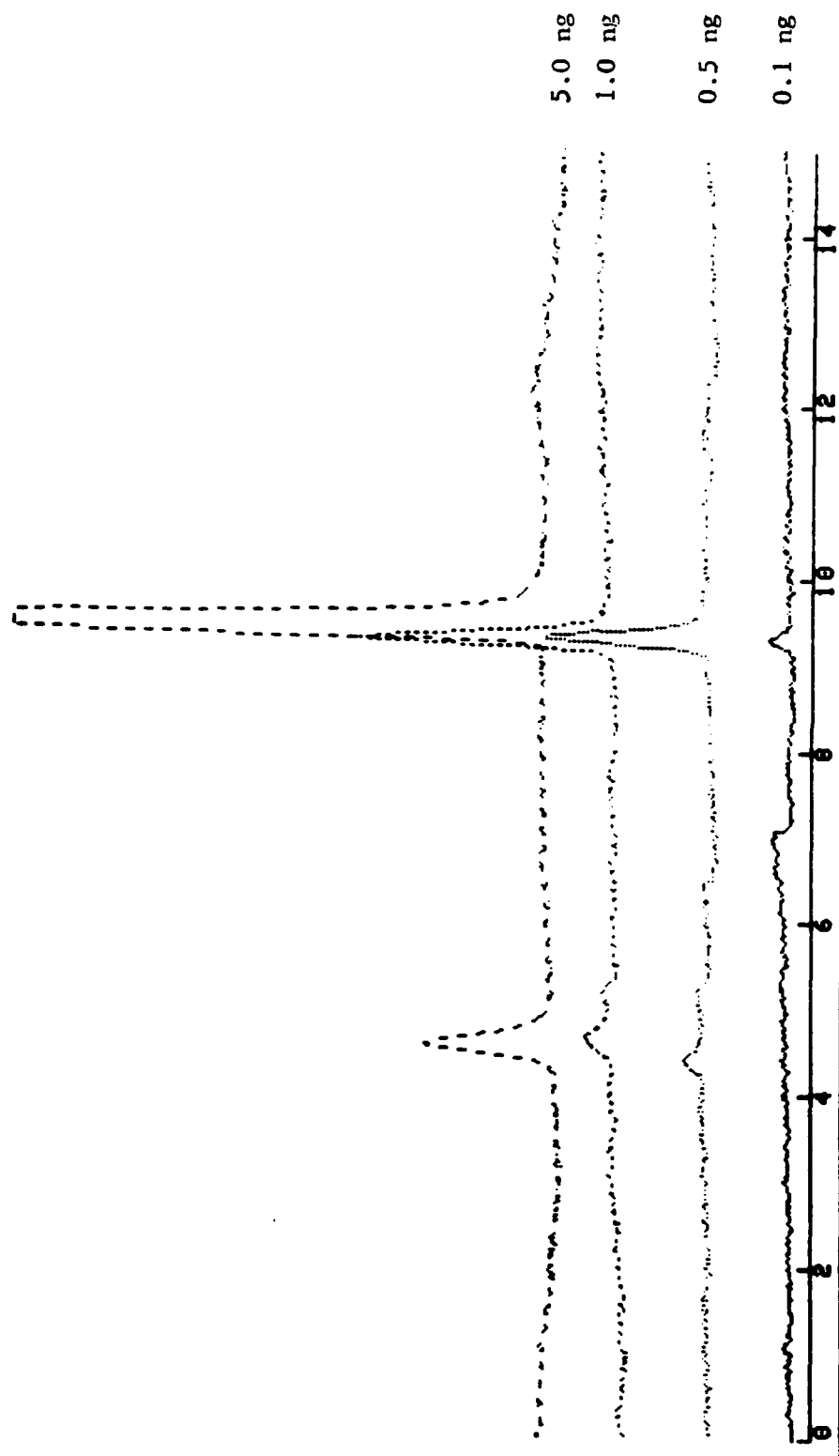


Figure 2. Detection of STX after oxidation and resolution by HPLC. The recorder responses are superimposed (overlay) in a linear fashion and represent a total of 5, 1, 0.5, and 0.1 ng of STX. The 5 ng injection saturated the detector and column and affected its retention time relative to that of the other concentrations. Note the unknown component at Rf 4.5. The ordinate is shown in relative units i.e., 0 to 100%.

Figure 3. Detection of the oxidized saxitoxin product extracted from urine collected from rat # 7 (1 hr after treatment) dosed with 1 ug of STX. Note the unknown reaction product at Rf 4.5 which is identical to that found in the STX standard.

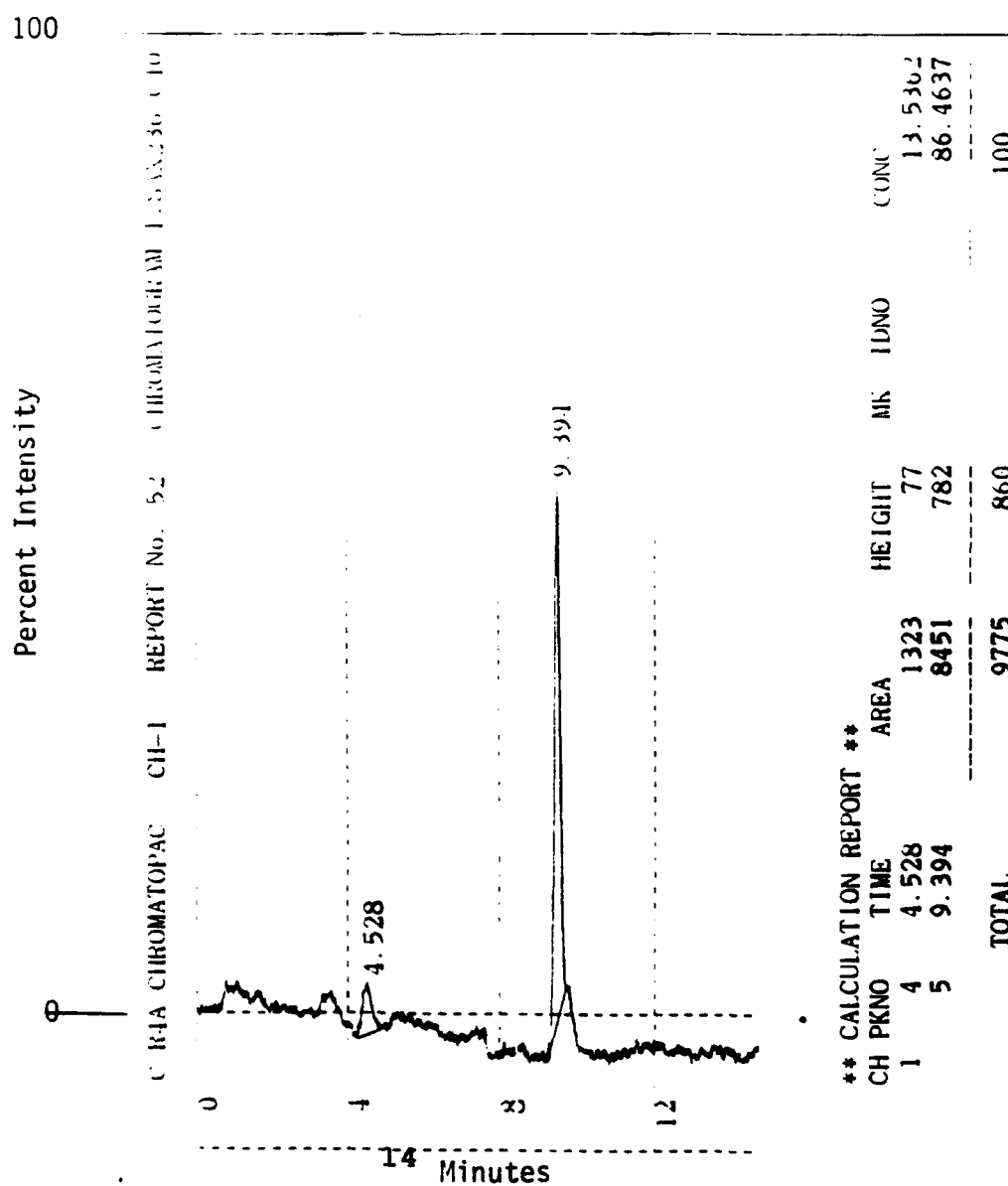
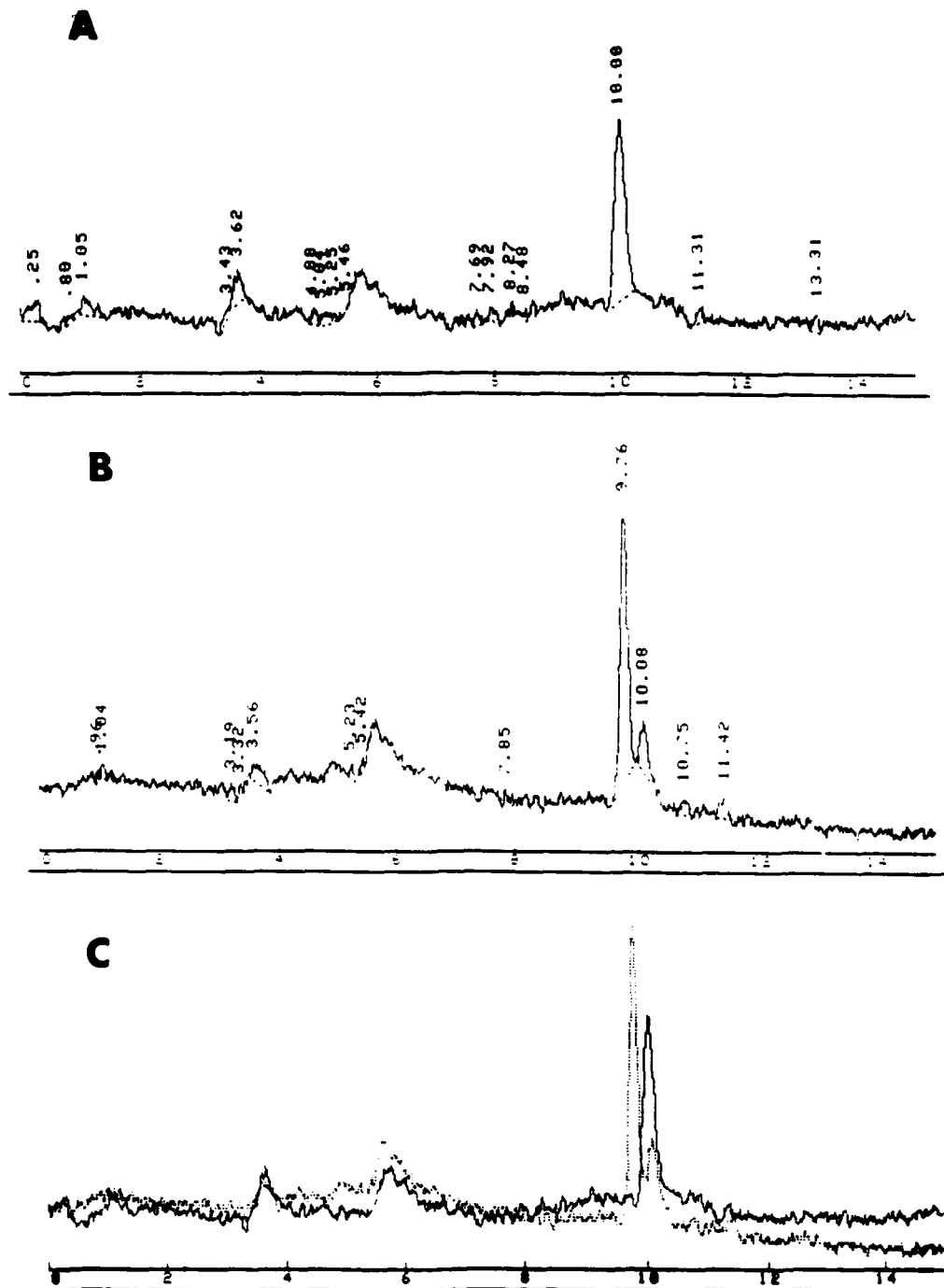


Figure 4-ABC. (A) Oxidation product (retention time 10.0) found in the urine of control rats (not treated with STX) when analyzed by HPLC. (B) Oxidation product of STX (retention time 9.7) found in urine of rat #5 collected in the 8th hour of the first day of treatment. The concentration of STX was 85.4 ng/ml. (C) Overlay of chromatograms of the control urine found in A and the STX treatment in B show complete resolution of STX. The ordinate represents percent relative intensity (0-100%).



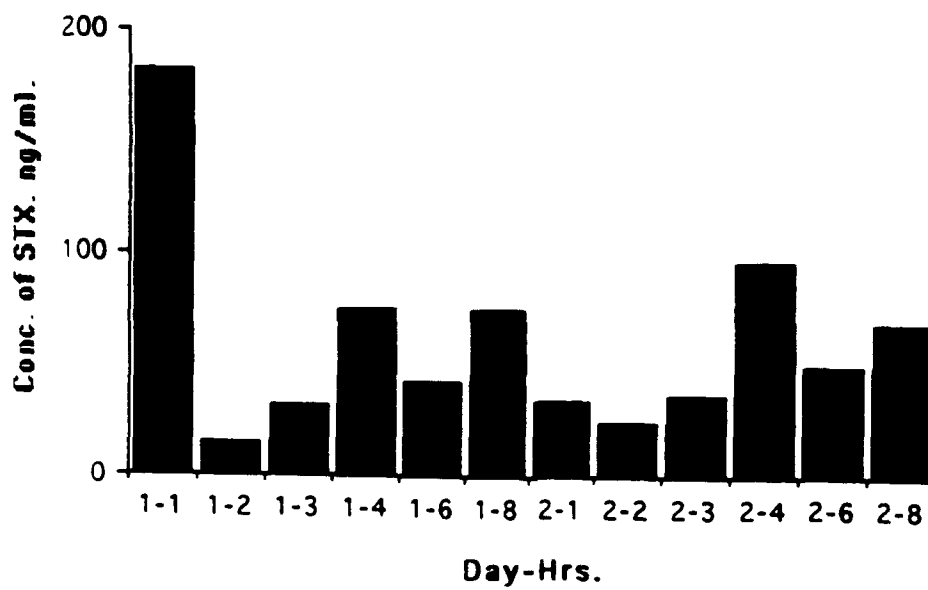


Figure 5. Average concentration (ng/ml) of STX found in the urine of three rats (# 5, 6, and 7) dosed with 1 ug per 200-gram rat. Each rat represents a separate experiment

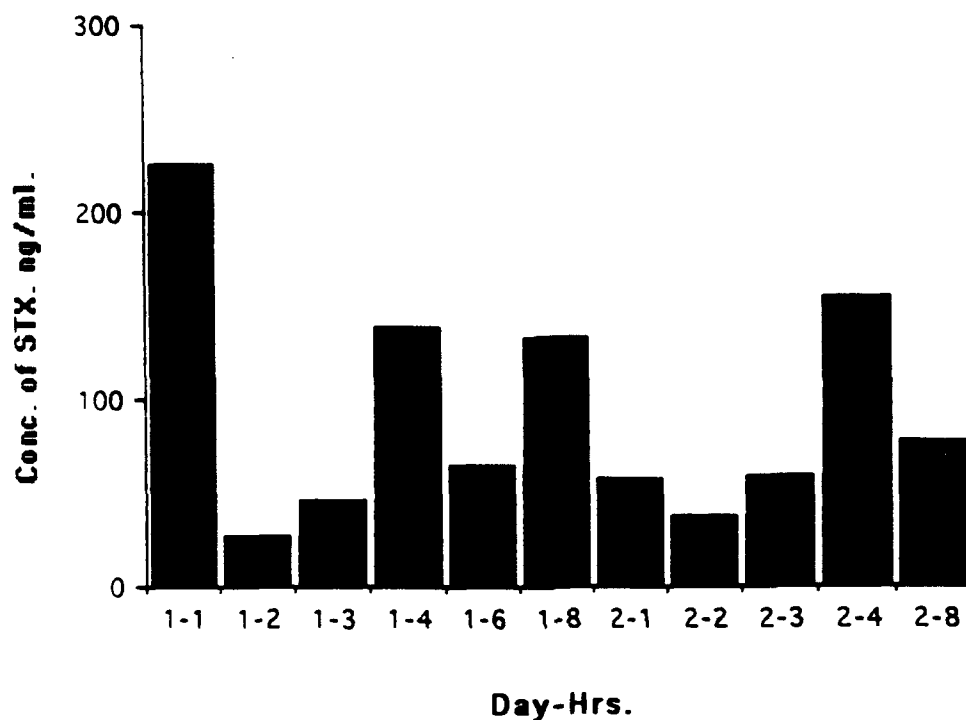


Figure 6. Concentration of STX (ng/ml) in urine of rat # 7 collected at 1, 2, 3, 4, 6 and 8 hrs after dosing with 1 ug STX on days 1 and 2.

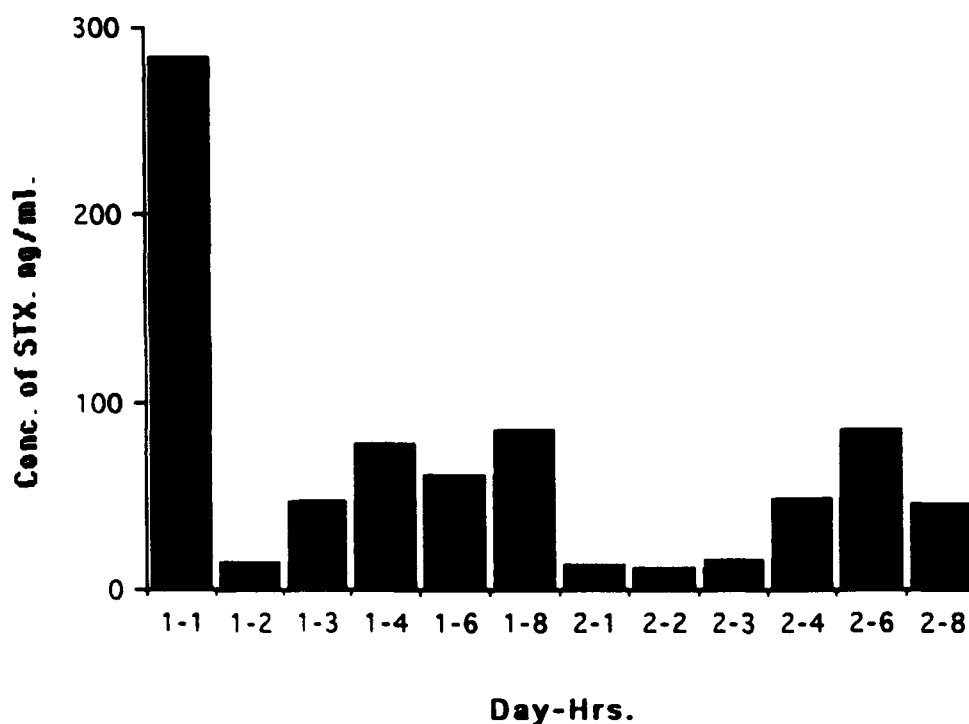


Figure 7. Concentration of STX (ng/ml) in urine of rat # 5 collected at 1, 2, 3, 4, 6 and 8 hours after dosing with 1 ug STX on days 1 and 2. Note the similarity in pattern to rat # 7.

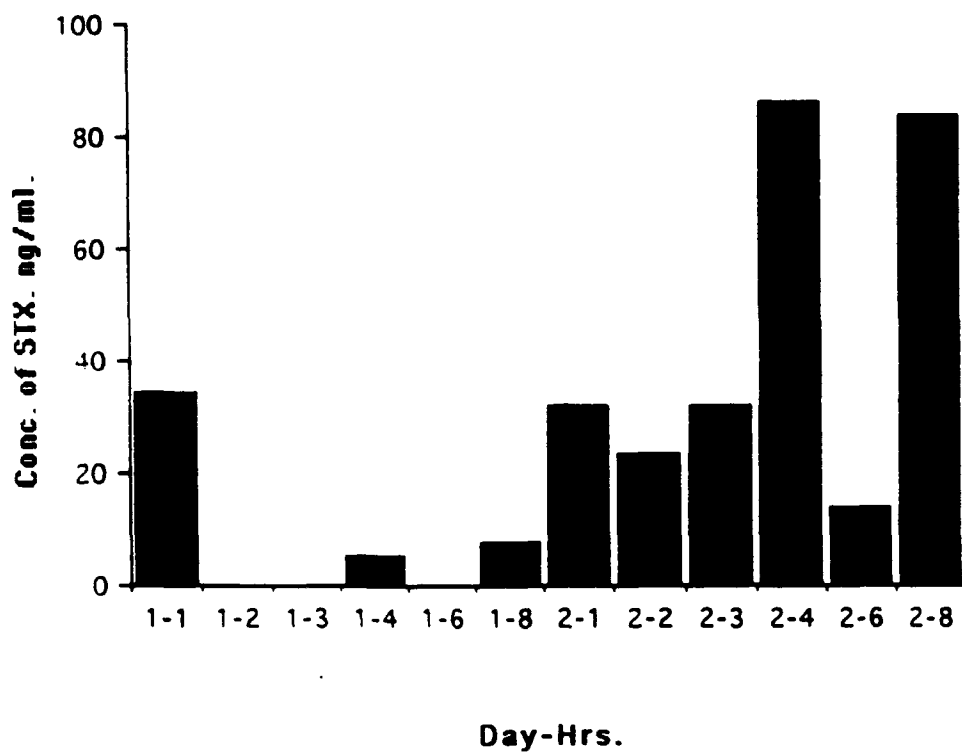


Figure 8. Concentration of STX (ng/ml) in urine of rat # 6 collected at 1, 2, 3, 4, 6 and 8 hrs after dosing with 1 ug STX on days 1 and 2. Note the large amount of STX found in the urine 4 and 8 hrs after treatment on day 2.

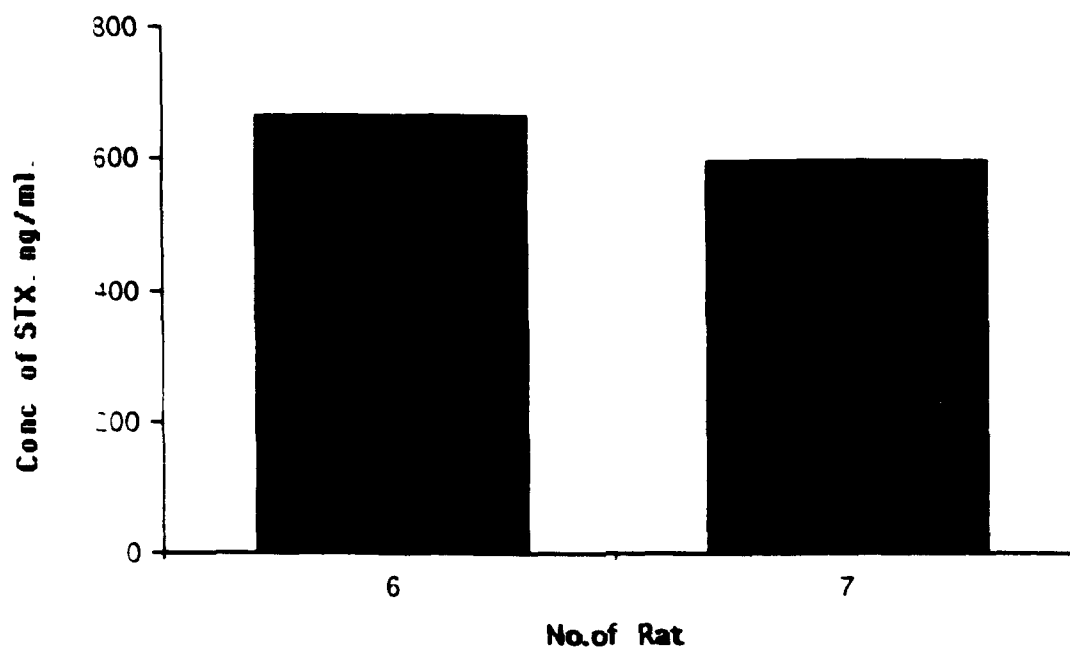


Figure 9. Concentration (ng/ml) of STX found in urine of rats #6 and #7 after dosing with STX (4 ug) on day 3. The animals died after 3 minutes and the urine was collected by 10 min.

Table 1. Concentration of saxitoxin (ng/ml) in rat urine at various time intervals after dosing (i. p.) of rats with 1 ug of saxitoxin on day 1 and 2 and concentration of STX found on day 3 after 4 ug dosing

Concentration of saxitoxin in urine. (ng/ml)					
Time (day-hr.)	Rat #5	Rat #6	Rat #7	Average	\pm Sd
1-1	284.8	34.2	226.1	181.7	107.0
1-2	14.0	ND**	27.8	13.9	11.4
1-3	47.4	ND**	45.9	31.1	22.0
1-4	78.0	5.3	139.0	74.1	33.4
1-6	61.8	ND**	64.1	42.0	29.7
1-8	85.4	7.6	132.6	75.2	37.9
2-1	12.1	32.0	57.5	33.9	18.6
2-2	11.3	23.1	37.7	24.0	10.8
2-3	16.2	32.0	59.0	35.7	17.7
2-4	48.2	86.0	154.9	96.4	44.2
2-6	85.1	13.7	N/A	49.4	35.7
2-8	45.8	83.9	76.9	68.9	16.6
3-0.1*	N/A	665.0	596.5	630.8	34.3

* rat dosed with 4ug STX at 0900 on day 3

** not detected amount of STX

Table 2. Total of saxitoxin (ng/ml) in rat urine at various time intervals after dosing (i. p.) of rats with 1 ug of saxitoxin on day 1 and 2 and total STX found on day 3 after 4 ug dosing.

Time (day-hr.)	Total of Saxitoxin (ng)				
	Rat #5	Rat #6	Rat #7	Average	\pm Sd
1-1	310.5	13.4	108.5	144.1	123.9
1-2	2.8	ND**	6.7	3.2	2.7
1-3	13.3	ND**	6.9	6.7	5.4
1-4	60.1	0.4	95.9	52.1	39.4
1-6	26.0	ND**	25.6	17.2	12.2
1-8	24.8	2.6	10.6	12.7	9.2
2-1	3.6	8.0	11.5	7.7	3.2
2-2	2.8	6.7	30.9	13.4	12.4
2-3	2.8	9.0	11.2	7.6	3.6
2-4	11.1	17.2	83.6	37.3	32.9
2-6	17.9	3.9	N/A	10.9	7.0
2-8	4.6	8.4	29.2	14.1	10.8
3-0.1 *	N/A	20.0	1.8	30.9	10.9

* rat doosed with 4ug STX at 0900 on day 3

** not detected amount of STX

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